

Analysis of Predicted Carbohydrate Transport Systems Encoded by *Bifidobacterium bifidum* PRL2010

Francesca Turrone,^{a,b} Francesco Strati,^a Elena Foroni,^a Fausta Serafini,^a Sabrina Duranti,^a Douwe van Sinderen,^b and Marco Ventura^a

Laboratory of Probiogenomics, Department of Genetics, Biology of Microorganisms, Anthropology and Evolution, University of Parma, Parma, Italy,^a and Alimentary Pharmabiotic Centre and Department of Microbiology, Bioscience Institute, National University of Ireland, Cork, Ireland^b

The *Bifidobacterium bifidum* PRL2010 genome encodes a relatively small set of predicted carbohydrate transporters. Growth experiments and transcriptome analyses of *B. bifidum* PRL2010 revealed that carbohydrate utilization in this microorganism appears to be restricted to a relatively low number of carbohydrates.

Members of the genus *Bifidobacterium* are arguably the most prominent bacterial representatives of the infant gut microbiota (27, 29, 33). Carbohydrates make up a major part of the human diet and are metabolized by both the host and the microbiota residing in the gastrointestinal tract (GIT). Carbohydrate availability is claimed to represent a determining factor that has shaped the genomes of the microbiota that live in the distal part of the intestine (9, 32). Thus, it is important to precisely understand which carbohydrates and how they are metabolized by enteric microorganisms, such as bifidobacteria, and how this capacity may endow bifidobacteria with a competitive edge in this niche.

Although a significant amount of information is available concerning bifidobacterial glycosyl hydrolases, i.e., enzymes involved in the breakdown of carbohydrates (for reviews, see references 14 and 31), little is known about carbohydrate transporters encoded by this group of microorganisms. One relevant finding in this respect came from bifidobacterial genome analysis, which revealed that bifidobacteria mainly perform carbohydrate transport by means of members of the ATP binding cassette (ABC) family but employ relatively few, if any, phosphoenolpyruvate-phosphotransferase (PEP-PTS) systems (10; for a review, see reference 11).

Genomic approaches have in recent times governed investigations regarding the mechanisms by which bifidobacteria colonize the human gut (7, 12, 34). In a recent study, we discovered that the genome of *Bifidobacterium bifidum* PRL2010 harbors a specific set of genes involved in the utilization of host-derived glycans, in particular those present in mucins that represent the outermost layer of the intestinal mucosa (24, 28). These findings therefore highlight *B. bifidum* PRL2010's capacity to colonize the human intestine (24, 28). To further understand how carbohydrate utilization contributes to host colonization, we investigated carbohydrate uptake features encoded by *B. bifidum* PRL2010 and compared such features to those of other bifidobacteria.

Evaluation of the growth profile of *B. bifidum* on different carbohydrates. Bifidobacterial strains used in this study were routinely cultivated in an anaerobic atmosphere (2.99% H₂, 17.01% CO₂, and 80% N₂) in a chamber (Concept 400; Ruskin) at 37°C for 32 h in de Man-Rogosa-Sharpe (MRS) (Scharlau Chemie, Barcelona, Spain) medium supplemented with 0.05% (wt/vol) L-cysteine hydrochloride. Growth curves of bifidobacteria, performed in triplicate, were obtained from cultures grown on a semisynthetic medium supplemented with various carbon sources, which included mono-, oligo-, and polysaccharides. Growth of *B. bifidum* PRL2010 was examined on 32 different carbohydrates, in-

cluding 20 carbon sources that had previously been tested (24). As shown in Table 1, *B. bifidum* PRL2010 is capable of growth on a variety of carbohydrates, which include various disaccharides, such as lactose, and amino sugars, such as *N*-acetylgalactosamine, as previously shown by Turrone et al. (24). No appreciable growth (i.e., where the final optical density at 600 nm [OD₆₀₀] was below 0.1) was observed on other sugars, such as mannitol, xylan, or α -cyclodextrin. Notably, the obtained carbohydrate utilization profile demonstrates growth on components of host-derived glycans, such as human milk oligosaccharides (HMOs) or mucin, as represented by lactose, galactose, *N*-acetylgalactosamine, and *N*-acetylglucosamine. These findings may thus be a direct reflection of the ecological niche in which this microorganism predominates, i.e., the infant colon. However, appreciable growth of *B. bifidum* PRL2010 (i.e., a final OD₆₀₀ of >0.6) was also noticed on certain plant-derived carbohydrates such as (iso)maltose, melibiose, sucrose, fructo oligosaccharides (FOS), (iso)maltulose, ribose, fructose, and turanose (Table 1). Compared to other bifidobacteria tested or data available in literature (13), and with the exception of *Bifidobacterium animalis* subsp. *lactis* BB12, it seems that *B. bifidum* PRL2010 is able to metabolize a somewhat lower number of carbohydrates (only eight) that sustain reasonable good growth (i.e., a final OD₆₀₀ of >1.2) (Table 1). However, these data require careful interpretation, as they represent a modest selection of carbohydrates and bifidobacterial strains, and therefore, no solid conclusions can be drawn from this.

Genomics of carbohydrate transport systems. A genome sequence survey of *B. bifidum* PRL2010 employing Artemis software (18) for the presence of genes that encode predicted carbohydrate transporters returned a substantial number of such genes, although this number was lower than that obtained from genome searches of other human enteric bifidobacteria, such as *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium adolescentis*, and *Bifidobacterium breve* (6, 12, 21). In fact, according to the Transporter Classification

Received 27 February 2012 Accepted 25 April 2012

Published ahead of print 4 May 2012

Address correspondence to Marco Ventura, marco.ventura@unipr.it.

Supplemental material for this article may be found at <http://aem.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.00629-12

TABLE 1 Growth capacity of *B. bifidum* PRL2010 and other bifidobacteria on various carbohydrates

Type of carbohydrate and carbon source	Growth of bifidobacterial strain ^a						
	<i>B. bifidum</i> PRL2010	<i>B. longum</i> subsp. <i>longum</i> NCC2705 ^b	<i>B. dentium</i> Bd1	<i>B. adolescentis</i> ATCC 15703	<i>B. breve</i> UCC2003	<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. animalis</i> subsp. <i>lactis</i> BB12
Monosaccharides							
Arabinose	+	++	++	+	—	—	—
Fructose	++	+	++	+++	+++	+++	+
Fucose	+	ND	—	+	+	++	+
Galactose	++	+++	++	++	+++	+++	+++
Glycerol	+	—	—	+	—	+	—
Glucose	+	+++	+++	+++	+++	+++	+++
Mannitol	—	—	++	+	—	+	—
Mannose	+	—	+++	—	+	++	+
<i>N</i> -Acetylgalactosamine	+++	ND	—	+	—	+	—
<i>N</i> -Acetylglucosamine	++	—	—	+	+	++	—
<i>N</i> -Acetylneuraminic acid	—	—	—	—	+	+	—
Ribose	++	+	+++	—	+++	++	++
Sorbitol	+	—	—	+++	—	+	—
Xylose	+	++	+	—	—	+	+
Di- and trisaccharides							
Isomaltose	++	ND	++	+++	++	++	++
Isomaltulose	++	ND	++	+++	++	+	+
Lactose	+++	+++	+++	++	+++	+++	++
Lactulose	+++	ND	+++	+++	+++	+++	—
Maltose	+++	++	+++	+++	+++	+++	+++
Maltulose	++	ND	++	++	++	+++	—
Melibiose	+++	++	++	+++	+++	+++	+++
Sucrose	+++	++	+++	+++	+++	+++	++
Trehalose	+	—	+	+	—	+	—
Turanose	++	ND	++	++	+++	++	+
Oligosaccharides and polysaccharides^c							
α -Cyclodextrin	—	ND	—	—	—	—	—
Arabinogalactan	+	+	+	+	—	+	—
Fructo oligosaccharides (FOS)	+++	++	+++	+++	+	+++	+
Galacto oligosaccharides (GOS)	+++	ND	+++	+	+++	+++	++
Inulin	+	ND	+	+++	—	++	—
Maltodextrin	+	ND	+++	+++	++	+++	++
Mucin	++	ND	+	—	—	—	—
Xylan	—	ND	+	—	—	—	—

^a Symbols: —, no growth as measured by $0.1 \leq OD_{600}$ or $10^5 \leq CFU/ml$; +, poor growth as measured by $0.1 < OD_{600} \leq 0.6$ or $10^5 < CFU/ml \leq 5 \times 10^6$; ++, good growth as measured by $0.6 < OD_{600} \leq 1.2$ or $5 \times 10^6 < CFU/ml \leq 5 \times 10^7$; +++, very good growth as measured by $OD_{600} > 1.2$ or $CFU/ml > 5 \times 10^7$. ND, not determined.

^b As indicated in the study of Parche et al. (13).

^c Carbohydrates were purified by the method of Rada et al. (16).

Database (TCDB; www.tcdb.org), just 25 genes (Fig. 1) are predicted to encode components of transport systems in the *B. bifidum* PRL2010 genome and dedicated to carbohydrate uptake, whereas the other human intestinal bifidobacterial genomes are predicted to contain between 35 and 68 such genes, as represented by *B. adolescentis* ATCC 15703 and *B. longum* subsp. *infantis* ATCC 15697, respectively (see Table S1 in the supplemental material). *B. animalis* subsp. *lactis* BB12 contains just 21 transporter genes, and this rather low number may have been due to genome simplification as a consequence of the continued cultivation of this strain in rather simple substrates during industrial processing. A similar scenario has been noticed for other strains used in the food industry (30).

When the analysis was extended to a nonenteric bifidobacterial strain, such as the oral cavity inhabitant *Bifidobacterium dentium*

Bd1, the relatively low number of identified carbohydrate transporters of *B. bifidum* PRL2010 is even more obvious (see Table S1 in the supplemental material). The 25 predicted carbohydrate transport proteins encoded by the PRL2010 genome can be classified into different groups: 8 belong to members of the ABC-type family, 12 are similar to various components of PEP-PTS systems, 1 is deduced to be a major intrinsic protein (MIP), while 4 represent secondary carriers, encompassing 2 members of the major facilitator superfamily (MFS), 1 member of the glycoside-pentoside-hexuronide (GPH):cation symporter family, and finally 1 member of the glucose/ribose porter family (GRP). The genes coding for the components of a given ABC transporter for a carbohydrate are commonly organized in a locus, typically encompassing between two and five genes that specify a sugar-specific solute binding protein (SBP), one or two permeases, and one or

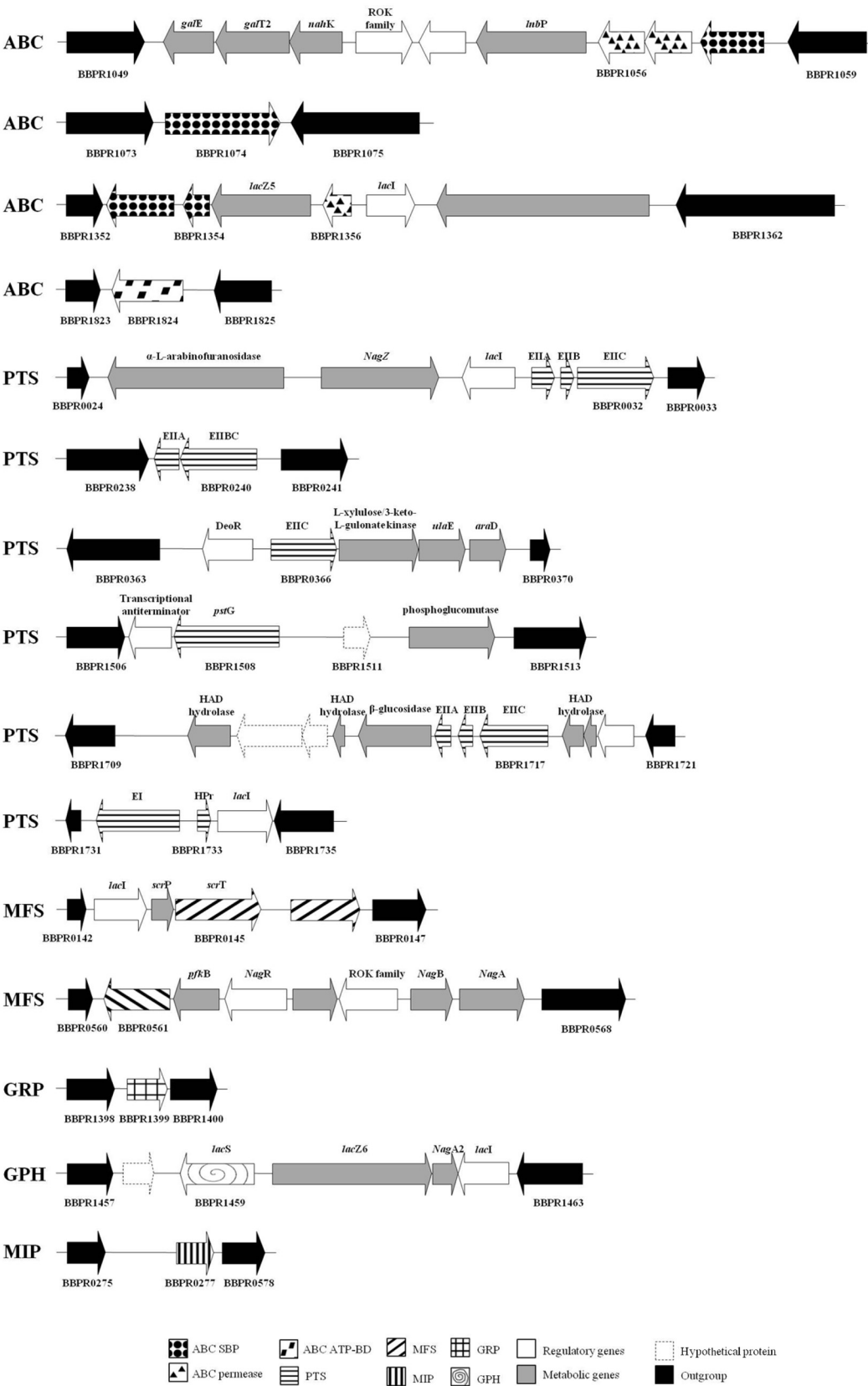
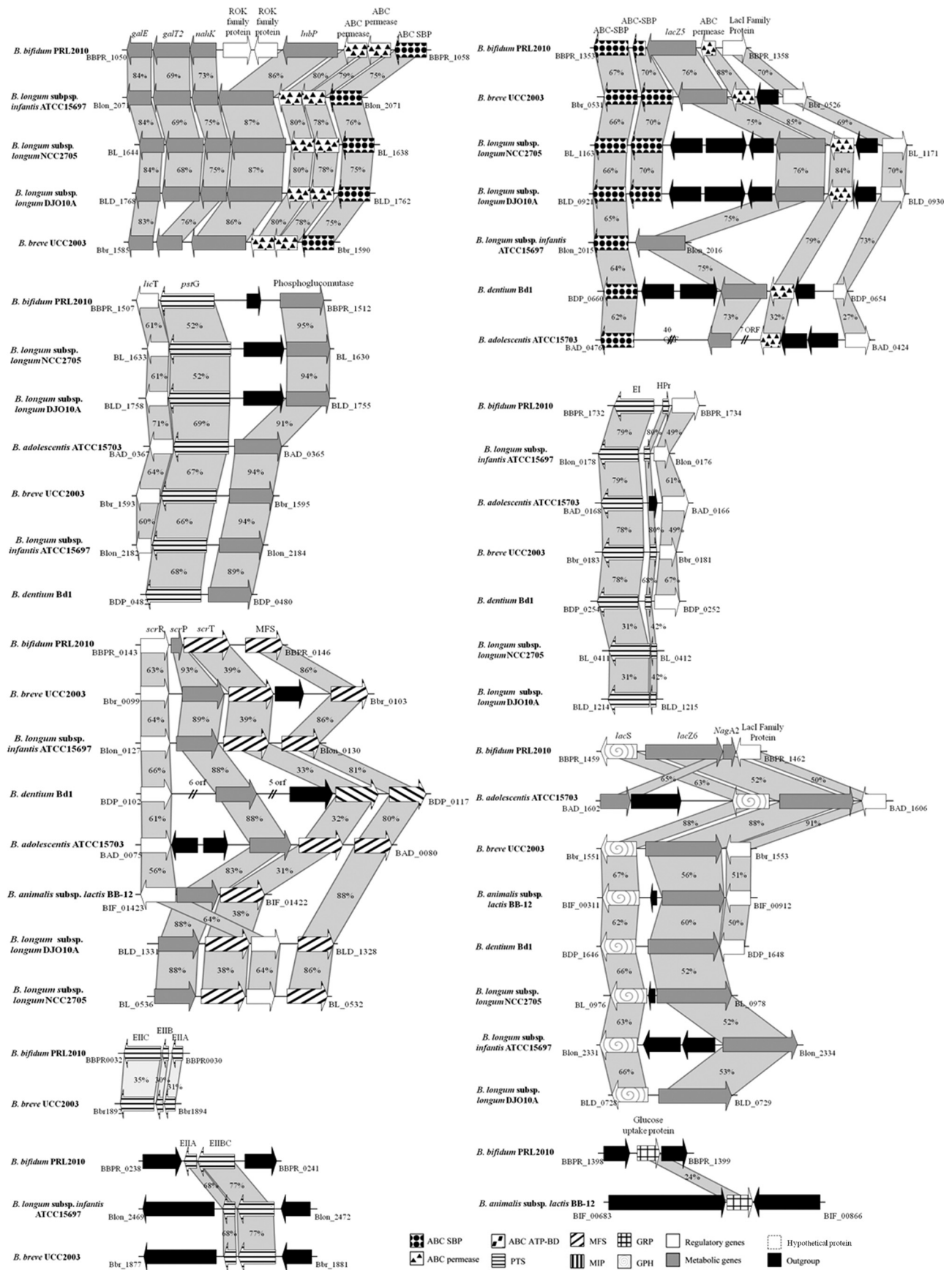


FIG 1 Genetic maps of the predicted carbohydrate transporter gene clusters identified in the genome of *B. bifidum* PRL2010. Each individual gene is represented by an arrow and is colored or marked according to the predicted function as indicated in the figure. HAD, haloacid dehalogenase.

TABLE 2 Carbohydrate transporters of *B. bifidum* PRL2010 used in RT-qPCR experiment

ORF	Predicted function	Family ^a	Homologous ORF ^b					Carbohydrate substrate ^c
			<i>B. longum</i> subsp. <i>longum</i> NCC2705	<i>B. breve</i> UCC2003	<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. adolescentis</i> ATCC 15703	<i>B. dentium</i> Bd1	
BBPR_0032	PTS system, subunit IIC	4.A.7: the PTS L-ascorbate (L-Asc) family		Bbr_1892				Degradation product(s) of host glycans (e.g. mucin) and complex carbohydrates (e.g., FOS)
BBPR_0145	Sucrose-like transporter ScrT	2.A.1: the major facilitator superfamily (MFS)	BL_0534	Bbr_0101	Blon_0129	BAD_0079	BDP_0116	Disaccharides (e.g., turanose)
BBPR_0240	PTS system, subunit IIBC	4.A.1: the PTS glucose-glucoside (Glc) family		Bbr_1880	Blon_2471			Degradation product(s) of host glycans (e.g., N-acetylglucosamine) Pentoses (e.g., ribose)
BBPR_0366	PTS system, subunit IIC	4.A.5: the PTS galactitol (Gat) family						Monosaccharides (e.g., fucose, fructose)
BBPR_0561	L-Fucose permease	2.A.1.7: the fucose:H ⁺ symporter (FHS) family						Disaccharides (e.g., isomaltulose, maltulose, and threulose)
BBPR_1074	Solute binding protein of ABC transporter system	3.A.1.2: the carbohydrate uptake transporter-2 (CUT2) family	BL_1498	Bbr_1111	Blon_1050	BAD_0775	BDP_1208	Multiple carbohydrates (e.g., fructose, glucose, galactose, mucin, and N-acetylglucosamine)
BBPR_1056	ABC transporter permease	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1640	Bbr_1588	Blon_2175			Multiple carbohydrates (e.g., fructose, glucose, galactose, mucin, and N-acetylglucosamine)
BBPR_1057	ABC transporter permease	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1639	Bbr_1589	Blon_2176			Multiple carbohydrates (e.g., fructose, glucose, galactose, mucin, and N-acetylglucosamine)
BBPR_1058	ABC transporter solute binding protein	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1638	Bbr_1590	Blon_2177			Multiple carbohydrates (e.g., fructose, glucose, galactose, mucin, and N-acetylglucosamine)
BBPR_1353	Solute binding protein of ABC transporter system	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1163	Bbr_0531	Blon_2015	BAD_0476	BDP_0660	Degradation product(s) of host glycans
BBPR_1356	ABC transporter permease	3.A.1.1: the carbohydrate uptake transporter-1 (CUT1) family	BL_1169	Bbr_0528	Blon_0342	BAD_0427	BDP_0656	Degradation product(s) of host glycans
BBPR_1399	Glucose uptake protein	2.A.7.5: the glucose/ribose porter (GRP) family						Monosaccharides (e.g., ribose and glucose)
BBPR_1459	Galactoside symporter	2.A.2: the glycoside-pentoxide-hexuronide (GPH):cation symporter family	BL_0976	Bbr_1551	Blon_2331	BAD_1608	BDP_1646	Galactose, lactose, and GOS
BBPR_1508	PTS system glucose-specific transporter subunit IIA	4.A.1: the PTS glucose-glucoside (Glc) family	BL_1632	Bbr_1594	Blon_2183	BAD_0366	BDP_0482	Glucose
BBPR_1715	PTS system cellobiose-specific transporter subunit IIA	4.A.3: the PTS lactose-NAN'-diacetylchitobiose-β-glucoside (Lac) family						Disaccharides (e.g., turanose and trehalose)
BBPR_1717	PTS system cellobiose-specific transporter subunit IIC	4.A.3: the PTS lactose-NAN'-diacetylchitobiose-β-glucoside (Lac) family						Disaccharides (e.g., turanose and trehalose)

^a According to the TCDB Database (20).^b Similarity with other carbohydrate transporters identified in the currently available bifidobacterial genome sequences (NCBI source). The percentage similarity used to determine whether these transporters are also encoded by other bifidobacteria is above 30% at the amino acid level with an E value below E-4 (0.0001).^c The prediction was based on the results achieved by RT-qPCR and global genome transcription profiling. Carbohydrates enhancing expression of the gene were tested.



two predicted ATPase proteins, although such ATPases can be shared among different ABC transporters and the corresponding gene(s) can therefore be absent from such a locus (4). We identified two loci that encode putative carbohydrate uptake ABC-type systems (represented by BBPR_1056 to BBPR_1058, BBPR_1353–BBPR_1354, and BBPR_1356), made up of genes encoding three SBPs (BBPR_1353, BBPR_1354, and BBPR_1058) and three permeases (BBPR_1056, BBPR_1057, and BBPR_1356). Hydropathy profile analysis using the ExPaSy Proteomic Server predicts that PRL2010-encoded permease proteins and SBPs contain six transmembrane domains and one transmembrane domain, respectively (see Fig. S1 in the supplemental material), which is typical for permease and SBP proteins of Gram-positive bacteria (4). Furthermore, the genome of PRL2010 contains a single predicted ATPase-encoding gene (BBPR_1824) as well as an additional SBP-encoding gene (BBPR_1074), neither of which is located next to other genes that specify one or more components of an ABC family carbohydrate uptake system (1, 13). We found a similarity above 60% at the amino acid level (extended to key amino acid residues sustaining enzymatic activities) between the deduced product of BBPR_1824 and that of BL0673 of *B. longum* subsp. *longum* NCC2705 and MsiK of *Streptomyces coelicolor* A3, both of which are believed to encode a universal ATPase for ABC-type carbohydrate transporters identified in these microorganisms (1, 13). These findings suggest that the functionality of the two identified PRL2010 ABC-type carbohydrate uptake systems rely on this single predicted ATPase-encoding gene (Fig. 1). Based on BLAST analysis, the four putative secondary transporters that belong to the MFS, GPH, and GRP families, including proton facilitators and symporters, are expected to transport mono- and disaccharides (Table 2). All these secondary transporters are predicted to consist of a single integral membrane-associated protein, which traverses the membrane 10 to 12 times (Fig. S1).

The genome of PRL2010 also contains four complete PTS systems, represented by the general components histidine protein (HPr) and enzyme I (EI), and four sets of the variable components EIIA, EIIB, and EIIC (see Fig. S1 in the supplemental material).

The organization and location of the carbohydrate transporter-encoding genes identified in the genome of *B. bifidum* PRL2010 and their closest homologs from all other publicly available bifidobacterial genomes are schematically represented in Fig. 2, where the deduced amino acid sequences of the PRL2010 carbohydrate transporter loci are aligned with homologs of other bifidobacteria. This comparative analysis revealed a high level of conservation (>70% identity) between the ABC permeases, SBP, and some of the PTS component proteins found in various bifidobacteria. In contrast, the EIIA, EIIB, and EIIC enzymes encoded by BBPR_1715, BBPR_1716, and BBPR_1717, respectively, and predicted to specify a complete PTS system, and BBPR_0366, which encodes a membrane-embedded EIIC carbohydrate transport component, only appeared to be present in the genomes of strains of *B. bifidum*, thus apparently representing unique genetic features of this species and supporting the notion that such transporters may provide a specific phenotype or particular niche access to *B. bifidum* strains.

Notably, many of the gene clusters involved in carbohydrate transport contain genes encoding putative regulators, which may indicate that such gene clusters are subject to substrate-dependent regulation in a similar fashion as previously shown for other bifidobacterial carbohydrate metabolism loci (14, 23). The genes specifying putative ABC-type permeases as well as PTS systems were indeed flanked by genes encoding such predicted regulators such as carbohydrate kinases belonging to the ROK (repressor, open reading frame, kinase) family (BBPR_1053–BBPR_1054), a transcription antiterminator of the BglG type (BBPR_1507), a DeoR-type regulator (BBPR_0365), and LacI-type regulators (BBPR_0029 and BBPR_1734) (Fig. 1). Similarly, the predicted MFS- and GPH-encoding genes present in the PRL2010 genome are located close to genes specifying putative regulators of the LacI type (BBPR_0143 and BBPR_1462) or belonging to the ROK family (BBPR_0565) (Fig. 1).

Phylogenetic analysis based on SBP protein sequences. In order to assess the distribution of SBP homologs across the *Bifidobacterium* genus, we surveyed available bifidobacterial genomic data for such genes, whose protein products were then aligned using ClustalW to produce an unrooted neighbor-joining phylogenetic tree, which was built using PHYLIP (Phylogeny Inference Package, version 3.5c) (5) (see Fig. S2 in the supplemental material). All analyzed SBPs, except for the *B. bifidum* PRL2010 SBP encoded by BBPR_1074, were shown to be closely related to members of the families Sbp_bac_1 and Sbp_bac_3 (22). Thus, BBPR_1074 might encode a novel solute binding protein never identified previously in bifidobacteria and consequently suggesting the existence of a novel family of SBP.

Transcriptional analysis of carbohydrate transporter loci. In order to determine whether the genes encoding the predicted carbohydrate transporters, including components of the ABC systems, PTS systems, and secondary carriers (MFS, GPH, and GRP), as identified on the *B. bifidum* PRL2010 genome are differentially transcribed when PRL2010 is cultivated on different carbohydrate growth substrates, we evaluated the level of predicted transporter gene-specific mRNAs by quantitative real-time PCR (RT-qPCR) assays according to the guidelines of Bustin et al. (2). Such experiments were performed using mRNA samples extracted from three independent exponentially growing cultures of *B. bifidum* PRL2010, which had been resuspended in prewarmed MRS medium containing one of a varied set of carbohydrates. Aliquots of 20 ml of PRL2010 cultures were centrifuged for 10 min at 4,000 × g at 4°C in the presence of RNA-later (Ambion). The pellets were then immediately frozen in liquid nitrogen and submitted to RNA extraction using a previously described method, which includes a DNase treatment (35). The quality and integrity of the RNA were checked by Experion (Bio-Rad) analysis. cDNA was synthesized and purified using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to the supplier's instructions. RT-qPCR primers used including those for normalization are described in Table 3. Primers used for normalization were those previously described (25). Criteria for primer design were a desired melting temperature (T_m) value between 58 and 60°C and an amplicon size of approximately 100 to 200 bp. RT-qPCR was performed

FIG 2 Schematic comparative representation of carbohydrate transporter-encoding genes of *B. bifidum* PRL2010 and of various other bifidobacterial strains such as *B. dentium* Bd1, *B. adolescentis* ATCC 15703, *B. breve* UCC2003, *B. longum* subsp. *longum* NCC2705, *B. longum* subsp. *longum* DJO10A, *B. longum* subsp. *infantis* ATCC 15697, and *B. animalis* subsp. *lactis* BB12. Each arrow indicates an open reading frame (ORF), the size of which is proportional to the length of the arrow. Predicted protein function is indicated above each arrow. The corresponding amino acid identity as a percentage is indicated.

TABLE 3 Primer information related to genes targeted in RT-qPCR experiments

Type of gene targeted and primer ^a	Sequence	Melting temp (°C)	Product size (bp)	Targeted ORF/gene
Target genes				
A1056Fw	CCCCAGTACTCGATCTTCCA	60.07	192	BBPR_1056
A1056Rev	CAGCACGATGGTGAAGAAAA	59.84		BBPR_1056
A1057Fw	GTCGGATTCCAGAACTTCCA	60.05	164	BBPR_1057
A1057Rev	AAACCGGTAACGTGCTTGAC	60.04		BBPR_1057
A1058Fw	AAACGACTGCTTGCTGGTCT	60.05	201	BBPR_1058
A1058Rev	GATGTTCCGGTTGTCTCTGT	59.84		BBPR_1058
A1353Fw	TGACGCCAGCTTCTACAATG	60.01	172	BBPR_1353
A1353Rev	TTTGAGATGGGTGTTGACCA	59.94		BBPR_1353
A1354Fw	GAGACGGTCACCATCAACCT	59.97	168	BBPR_1354
A1354Rev	CTTGACGGGTAGCCCTGAT	60.13		BBPR_1354
A1356Fw	TCGTCTCCATGCTCATCAAC	59.79	103	BBPR_1356
A1356Rev	GTCACGATGTTGTCCACAG	60		BBPR_1356
P0030Fw	GATCGAGACGGTCGAGAAAA	60.34	174	BBPR_0030
P0030Rev	AGACCGATGACCAGCGATAC	60.1		BBPR_0030
P0032Fw	ACACCCTCGGCTACATTGTC	60	159	BBPR_0032
P0032Rev	CGGAGAAGACCAGGTACAGC	59.87		BBPR_0032
P0239Fw	AGATTACCACGGTCGCTCAG	60.28	188	BBPR_0239
P0239Rev	CTTGACCACATTCCAGTCCA	59.52		BBPR_0239
P0240Fw	CGATGACGATAACGACGATG	60.1	146	BBPR_0240
P0240Rev	GGGTGATGCAGTTCTCGATT	60.08		BBPR_0240
P1715Fw	ACCCCTTGACATGGAAGTGCT	59.58	135	BBPR_1715
P1715Rev	GTCGCCGGAATTCATCTTC	60.76		BBPR_1715
P1717Fw	CTCGTGGGTCTACACCACCT	60.03	152	BBPR_1717
P1717Rev	AGACCTGGCACACCTGGTAG	60.17		BBPR_1717
P1508Fw	GGTGTTTCATCAAGGCCATCT	59.93	164	BBPR_1508
P1508Rev	TCCACTTTTCGGAGTCCATC	60.05		BBPR_1508
P0366Fw	TCAACCTGCTGCTTGACAAC	60.03	185	BBPR_0366
P0366Rev	GAGCTTGCAGAACACCATGA	59.99		BBPR_0366
M0561Fw	AAACCACCAAGCCAGTCAAC	60.01	189	BBPR_0561
M0561Rev	GCACGGAGAAGATCAGGAAC	59.81		BBPR_0561
M1459Fw	CTCGTGGACAACACGAACAC	60.2	210	BBPR_1459
M1459Rev	CATGCCCCAGTAGGAGATGT	59.95		BBPR_1459
M0145Fw	ATGATCGGCCTGATTACGAC	59.92	145	BBPR_0145
M0145Rev	GATCCCTCCGAAGTCATCTG	59.61		BBPR_0145
M1399Fw	GGCAGGACAATCATCTCCTT	60.08	158	BBPR_1399
M1399Rev	GCCAGACCGTTCATCTCATT	60.08		BBPR_1399
A1074Fw	GCAGATGTGCCTGAAAATCA	59.81	153	BBPR_1074
A1074Rev	ATCAGCGTGTCTTTGAGGTT	60.02		BBPR_1074
Reference genes				
uvrD/Rep-F	ATACTCCGAGAATGCGGATG	60.06	165	<i>uvrD/rep</i>
uvrD/Rep-R	ACGACATCCCGCTCATATTC	59.92		<i>uvrD/rep</i>
pdxS-F	ATGATCAAGGGCATTTCAGGA	60.43	172	<i>pdxS</i>
pdxS-R	CGTCGAAGTGTCTTGTTCG	60.5		<i>pdxS</i>
gluC-F	CGCATCTCTCCGATATCCTC	59.76	184	<i>gluC</i>
gluC-R	ACAGACTCAGTCCCGTCACC	60.16		<i>gluC</i>
1653_fw	CTTCTCGTGGTGTCTCGGTAT	60.28	171	<i>atpB</i>
1653_rev	CGTGAAGAACAACGTCGAAA	59.88		<i>atpB</i>
1691_fw	GTGGATATACCCGGTCGATG	60.04	164	<i>glnD</i>
1691_rev	TAGTGACGGTCGTCATACCG	59.59		<i>glnD</i>
B.bif1	CCACATGATCGCATGTGATTG	63.31	278	16S rRNA
B.bif2	CCGAAGGCTTGCTCCCAA	65.76		16S rRNA

^a The direction of the primer is indicated at the end of the primer designation as follows: Fw and F, forward; Rev, R, and rev, reverse.

using the CFX96 system (Bio-Rad, Hercules, CA). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 95°C for 3 min, followed by 39 cycles, with 1 cycle consisting of 95°C for 5 s and 66°C for 20 s. The melting curve was measured from 65°C to 95°C with increments of 0.5°C/s.

Each PCR mix contained 12.5 µl of 2× SYBR SuperMix Green (Bio-Rad, Hercules, CA), 1 µl of cDNA dilution, each of the forward and reverse primers at 0.5 µM, and nuclease-free water to obtain a final volume of 20 µl. In each run, a negative control (no cDNA) for each primer set was included. The expression ratio of the selected genes was calculated and analyzed using the CFX

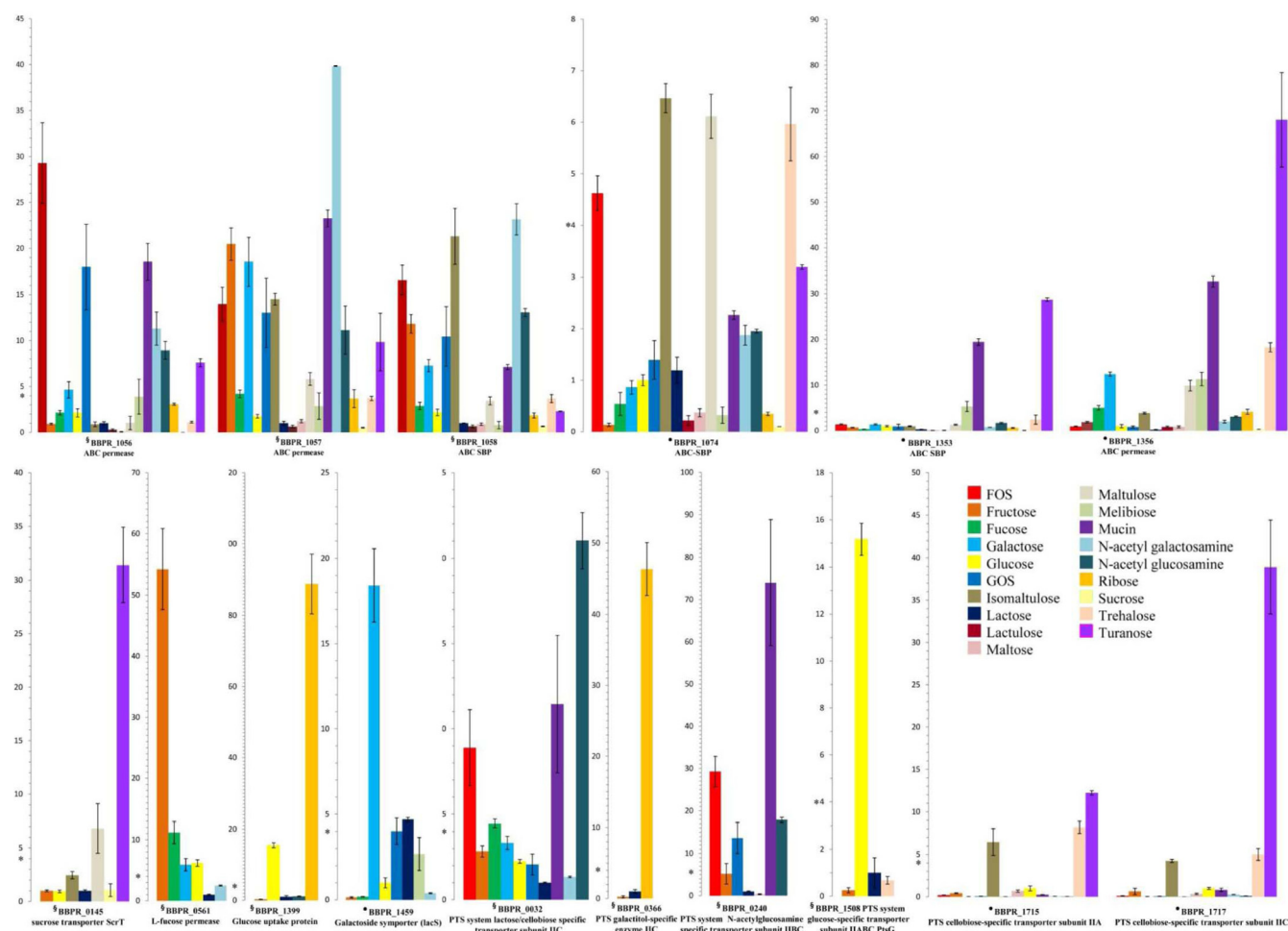


FIG 3 Relative transcription levels of carbohydrate transporter-encoding genes from *B. bifidum* PRL2010 upon cultivation in MRS medium supplemented with various carbon sources as analyzed by quantitative real-time PCR assays. The histograms indicate the relative amounts of the carbohydrate transporter-encoding gene mRNAs for the specific samples. The y axis indicates the fold induction of the investigated gene compared to the reference condition (lactose, which is indicated by §, or glucose, which is indicated by ●). The x axis represents the different carbohydrates tested for each ORF. In each panel, the ORF numbering indicates the gene code according to Fig. 1. Only those genes/carbohydrate conditions whose expression was significantly changed are shown. An asterisk on the y axis indicates that the change in fold induction is considered significant. The error bar for each column represent the standard deviation calculated from three replicates. GOS, galacto oligosaccharides.

Manager expression software (Bio-Rad, Hercules, CA). The cutoff value applied to highlight significant change in the expression was 4.

The mRNA levels corresponding to genes encoding individual components of identified *B. bifidum* PRL2010 carbohydrate transporters were shown to be variable in response to the various carbon sources used for growth (Fig. 3). Interestingly, the transcription of three predicted carbohydrate transport systems of PRL2010, i.e., the ABC locus (BBPR_1353 to BBPR_1356) (this presumed ABC-type transporter also exhibits induction upon growth in turanose [see below]) and two PTS loci (BBPR_0032 and BBPR_0240), was significantly enhanced when PRL2010 was cultivated on host-specific glycans such as mucin and host glycan constituents like *N*-acetylglucosamine (Fig. 3), and this may reflect a specialization of PRL2010 toward the particular ecological niche of the human GIT.

Furthermore, we identified genes that represent predicted carbohydrate transporter components and that were shown to exhibit specific transcriptional induction as a result of growth on

particular monosaccharides such as fructose (BBPR_0561), glucose (BBPR_1508), ribose (BBPR_0366 and BBPR_1399), galactose (BBPR_1459) or disaccharides such as the sucrose-like carbohydrate turanose (BBPR_0145 and BBPR_1715, BBPR_1717, and BBPR_1353 to BBPR_1356) (Fig. 4). Each of the genes that specify one of the ABC-type carbohydrate transporters, specified by BBPR_1056 to BBPR_1058, as well as the gene encoding SBP_{BBPR_1074} were shown to be induced when *B. bifidum* PRL2010 was grown on multiple carbon sources, reminiscent of what previously was described for several ABC systems identified in the genome of *B. longum* subsp. *longum* NCC2705 (8, 13). These data suggest that transcription of these transporters is subject to a common control mechanism and thus not necessarily regulated in response to their specific substrate.

Furthermore, relative expression levels between genes that are predicted to encode components of a particular carbohydrate transporter varied quite substantially when *B. bifidum* PRL2010 was grown on a specific carbohydrate. This finding is not unusual

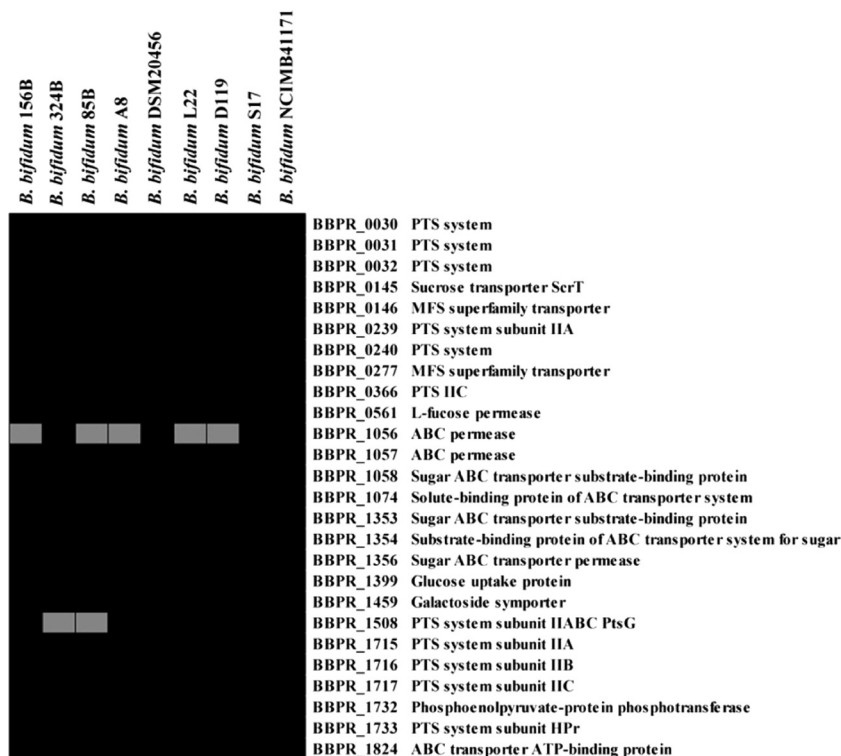


FIG 4 Identification of the conservation of carbohydrate transporter genes within several members of the *B. bifidum* taxon. The presence (black) or absence (gray) of key genes predicted to be involved in carbohydrate transporters in the currently publicly available *B. bifidum* genomes and on the basis of CGH data (24) is shown.

for SBP-encoding genes, because higher transcription is expected to lead to an abundance of SBP molecules (13), thus providing a more efficient substrate scavenging ability and consequent uptake. Since the SBPs of strain PRL2010 are encoded by distinct genes, we may envisage that multiple protein copies of SBP are cooperating with a corresponding carbohydrate permease, in a similar fashion to that described for other bacteria (3, 13, 15). The observation that a range of substrates can induce the expression of predicted SBP-encoding genes in *B. bifidum* PRL2010 may be linked to their low number in this genome with respect to other bifidobacterial genomes (e.g., 16 in *B. breve* UCC2003, 11 in *B. longum* subsp. *longum* NCC2705 and 20 in *B. longum* subsp. *infantis* ATCC 15697) and thus may be pivotal for the colonization strategy of PRL2010. Furthermore, it is tempting to speculate that PRL2010 has specialized itself in the metabolism of a relatively low number of carbohydrates and that the apparently low level of induction specificity of the SBP-encoding genes and other carbohydrate-transporting genes is just a consequence of this adaptation.

Evaluation of genes involved in carbohydrate transport in members of the *B. bifidum* species. In order to investigate the level of conservation of the genetic repertoire involved in carbohydrate transport within the *B. bifidum* taxon, we surveyed the currently publicly available *B. bifidum* genome sequences, i.e., *B. bifidum* S17 (36) and *B. bifidum* NCIMB 41171 (GenBank accession no. NZ_ABQP000000000) as well as previously published comparative genomic hybridization (CGH) data (24). The obtained data demonstrated that a large proportion of the 25 identified PRL2010 carbohydrate transport genes are conserved within these investigated members of the *B. bifidum* taxon. Only BBPR_1056 and

BBPR_1508 were found to be variably present in the tested *B. bifidum* strains (Fig. 4). Comparative analyses highlighted a high similarity (higher than 98% at nucleotide level) of these transporter genes with that identified in the genome of *B. bifidum* PRL2010, thus corroborating the CGH findings (Fig. 4).

Conclusions. This study provides the first detailed investigation into the genetic requirements for carbohydrate uptake in *B. bifidum* (Fig. 5). Our analysis of *B. bifidum* PRL2010 genes encoding predicted carbohydrate transporter systems revealed that, compared to other bifidobacterial strains residing in the infant gut, such as *B. longum* subsp. *infantis*, *B. breve*, and *B. longum* subsp. *longum* (17, 26, 27), PRL2010 contains a relatively limited number of such genes. Since this scenario is also found in two other *B. bifidum* strains for which genome sequences are available, it appears that in contrast to other bifidobacteria (for a review, see reference 14), carbohydrate breakdown/uptake in this bifidobacterial taxon is restricted to a comparatively low number of carbohydrates, which may represent an interesting genetic strategy for efficient colonization and survival in its ecological niche. The observed carbohydrate utilization profiles (Table 1) did not appear to reflect this specialization, but the majority of carbohydrates tested were mono- and disaccharides, which may have biased our analysis. Nevertheless, in contrast to other bifidobacteria, *B. bifidum* can utilize complex O-linked glycans associated with mucin, which are not commonly digested by other enteric bacteria, thus highlighting interesting and unique metabolic features of this taxon. The ability to rapidly retrieve a specific carbon source from a particular environment represents an important feature that would endow a bacterium with an undisputed ecological fitness

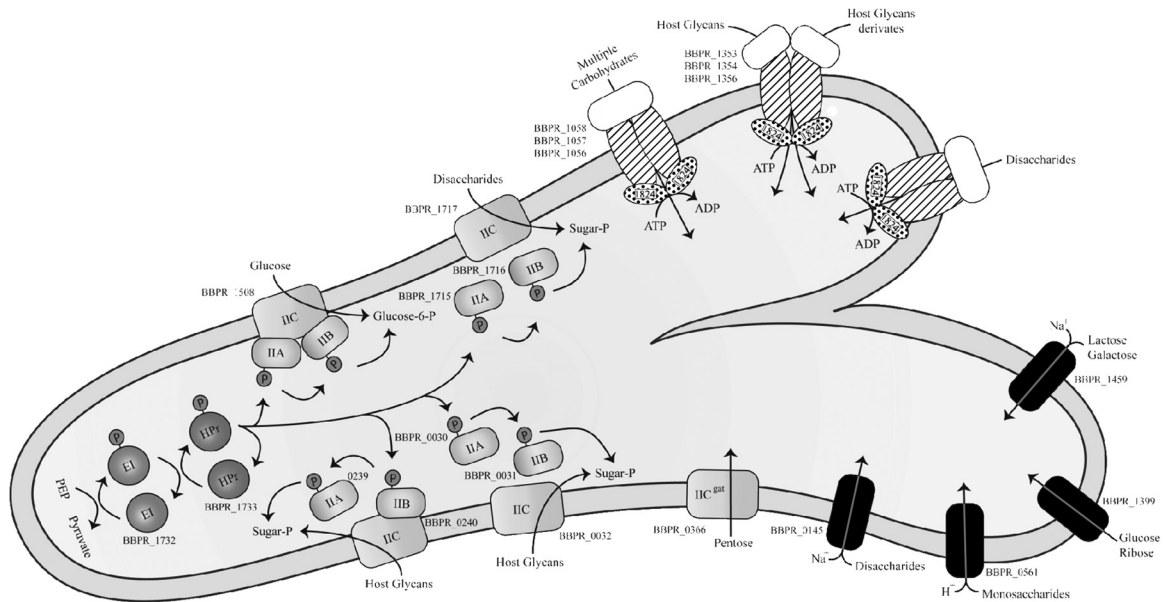


FIG 5 Carbohydrate transport systems of *B. bifidum* PRL2010. The ABC transporters are colored in white (predicted SBP subunit), gray (predicted permease subunit), or with dots (predicted ATPase subunit). The PTS transporters are depicted in gray, and secondary carriers are displayed in black. The derived putative substrates are inferred from *in silico* analyses in combination with experimental data. P, phosphate group.

(31). Furthermore, the main distinction between *B. bifidum* and other bifidobacteria is its capacity to grow on both HMOs and mucin, a capability that apparently provides a very strong selective advantage over other (bifido)bacteria. In fact, one could argue that because of this seemingly unique capability, it may have lost or did not acquire other carbohydrate-metabolizing abilities.

It is worth mentioning that the ABC-type transporters identified in *B. bifidum* genomes are not restricted to the transport of carbohydrates and also include predicted uptake systems for amino acids, peptides, metal ions (such as Mn^{2+} , Zn^{2+} , and Fe^{2+}), phosphate, lipoproteins, teichoic acid, as well as efflux systems for drugs such as nisin, macrolide, and anthracycline. Moreover, relative to other bifidobacterial genomes, a much larger proportion of the genes of the *B. bifidum* genome-encoded ABC-type transporters are dedicated to efflux rather than uptake (24, 28).

This report represents the first step toward understanding the complex nutritional relationships existing between the different members of the infant gut microbiota and toward elucidating the possible synergistic and antagonistic actions exerted by *B. bifidum* species with respect to other bacterial components residing in this ecosystem. Future investigations will be directed to explore how cocultivation of *B. bifidum* PRL2010 with other components of the infant gut microbiota may allow cross-feeding by the provision of unutilized mono- or disaccharides, which are derived from degraded diet or host polysaccharides and which may represent important growth substrates for such cross-feeding (bifido)bacteria.

ACKNOWLEDGMENTS

We thank Sacco srl and GenProbio srl for the financial support of the Laboratory of Probiogenomics. This work was financially supported by a FEMS Advanced Fellowship 2011 and an IRCSET Embark postdoctoral fellowship to F.T. D.V.S. is a member of The Alimentary Pharmabiotic Centre, which is a Centre for Science and Technology (CSET) funded by

the Science Foundation Ireland (SFI), through the Irish government's National Development Plans (grant 02/CE/B124 and 07/CE/B1368).

REFERENCES

1. Bertram R, et al. 2004. *In silico* and transcriptional analysis of carbohydrate uptake systems of *Streptomyces coelicolor* A3(2). J. Bacteriol. 186: 1362–1373.
2. Bustin SA, et al. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55: 611–622.
3. Davidson AL, Dassa E, Orelle C, Chen J. 2008. Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol. Mol. Biol. Rev. 72:317–364.
4. Driessen AJ, Rosen BP, Konings WN. 2000. Diversity of transport mechanisms: common structural principles. Trends Biochem. Sci. 25:397–401.
5. Felsenstein J. 1997. An alternating least squares approach to inferring phylogenies from pairwise distances. Syst. Biol. 46:101–111.
6. Lee JH, et al. 2008. Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. BMC Genomics 9:247. doi:10.1186/1471-2164-9-247.
7. Lee JH, O'Sullivan DJ. 2010. Genomic insights into bifidobacteria. Microbiol. Mol. Biol. Rev. 74:378–416.
8. Liu D, et al. 2011. Proteomics analysis of *Bifidobacterium longum* NCC2705 growing on glucose, fructose, mannose, xylose, ribose, and galactose. Proteomics 11:2628–2638.
9. Martens EC, Chiang HC, Gordon JI. 2008. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe 4:447–457.
10. Maze A, O'Connell-Motherway M, Fitzgerald GF, Deutscher J, van Sinderen D. 2007. Identification and characterization of a fructose phosphotransferase system in *Bifidobacterium breve* UCC2003. Appl. Environ. Microbiol. 73:545–553.
11. Nguyen TX, Yen MR, Barabote RD, Saier MH, Jr. 2006. Topological predictions for integral membrane permeases of the phosphoenolpyruvate:sugar phosphotransferase system. J. Mol. Microbiol. Biotechnol. 11: 345–360.
12. O'Connell Motherway M, et al. 2011. Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proc. Natl. Acad. Sci. U. S. A. 108:11217–11222.

13. Parche S, et al. 2007. Sugar transport systems of *Bifidobacterium longum* NCC2705. *J. Mol. Microbiol. Biotechnol.* 12:9–19.
14. Pokusaeva K, Fitzgerald GF, van Sinderen D. 2011. Carbohydrate metabolism in bifidobacteria. *Genes Nutr.* 6:285–306.
15. Quentin Y, Fichant G, Denizot F. 1999. Inventory, assembly and analysis of *Bacillus subtilis* ABC transport systems. *J. Mol. Biol.* 287:467–484.
16. Rada V, et al. 2008. Growth of infant faecal bifidobacteria and clostridia on prebiotic oligosaccharides in *in vitro* conditions. *Anaerobe* 14:205–208.
17. Roger LC, Costabile A, Holland DT, Hoyles L, McCartney AL. 2010. Examination of faecal *Bifidobacterium* populations in breast- and formula-fed infants during the first 18 months of life. *Microbiology* 156:3329–3341.
18. Rutherford K, et al. 2000. Artemis: sequence visualization and annotation. *Bioinformatics* 16:944–945.
19. Saier MH, Jr, Tran CV, Barabote RD. 2006. TCDB: the Transporter Classification Database for membrane transport protein analyses and information. *Nucleic Acids Res.* 34:D181–D186.
20. Saier MH, Jr, Yen MR, Noto K, Tamang DG, Elkan C. 2009. The Transporter Classification Database: recent advances. *Nucleic Acids Res.* 37:D274–D278.
21. Schell MA, et al. 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc. Natl. Acad. Sci. U. S. A.* 99:14422–14427.
22. Tam R, Saier MH, Jr. 1993. Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol. Rev.* 57:320–346.
23. Trindade MI, Abratt VR, Reid SJ. 2003. Induction of sucrose utilization genes from *Bifidobacterium lactis* by sucrose and raffinose. *Appl. Environ. Microbiol.* 69:24–32.
24. Turróni F, et al. 2010. Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc. Natl. Acad. Sci. U. S. A.* 107:19514–19519.
25. Turróni F, et al. 2011. Global genome transcription profiling of *Bifidobacterium bifidum* PRL2010 under *in vitro* conditions and identification of reference genes for quantitative real-time PCR. *Appl. Environ. Microbiol.* 77:8578–8587.
26. Turróni F, et al. 2009. Exploring the diversity of the bifidobacterial population in the human intestinal tract. *Appl. Environ. Microbiol.* 75:1534–1545.
27. Turróni F, et al. 2011. Ability of *Bifidobacterium breve* to grow on different types of milk: exploring the metabolism of milk through genome analysis. *Appl. Environ. Microbiol.* 77:7408–7417.
28. Turróni F, Milani C, van Sinderen D, Ventura M. 2011. Genetic strategies for mucin metabolism in *Bifidobacterium bifidum* PRL2010: an example of possible human-microbe co-evolution. *Gut Microbes* 2:183–189.
29. Turróni F, van Sinderen D, Ventura M. 2011. Genomics and ecological overview of the genus *Bifidobacterium*. *Int. J. Food Microbiol.* 149:37–44.
30. van de Guchte M, et al. 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proc. Natl. Acad. Sci. U. S. A.* 103:9274–9279.
31. van den Broek LA, Hinz SW, Beldman G, Vincken JP, Voragen AG. 2008. *Bifidobacterium* carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. *Mol. Nutr. Food Res.* 52:146–163.
32. Ventura M, Canchaya C, Fitzgerald GF, Gupta RS, van Sinderen D. 2007. Genomics as a means to understand bacterial phylogeny and ecological adaptation: the case of bifidobacteria. *Antonie Van Leeuwenhoek* 91:351–372.
33. Ventura M, et al. 2007. Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. *Microbiol. Mol. Biol. Rev.* 71:495–548.
34. Ventura M, et al. 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nat. Rev. Microbiol.* 7:61–71.
35. Ventura M, et al. 2005. The *clgR* protein regulates transcription of the *clpP* operon in *Bifidobacterium breve* UCC 2003. *J. Bacteriol.* 187:8411–8426.
36. Zhurina D, et al. 2011. Complete genome sequence of *Bifidobacterium bifidum* S17. *J. Bacteriol.* 193:301–302.